Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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SARS-CoV-2 in wastewater: From detection to evaluation

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ABSTRACT

SARS-CoV-2 presence in wastewater has been reported in several studies and has received widespread attention among the Wastewater-based epidemiology (WBE) community. Such studies can potentially be used as a proxy for early warning of potential COVID-19 outbreak, or as a mitigation measure for potential virus transmission via contaminated water. In this review, we summarized the latest understanding on the detection, concentration, and evaluation of SARS-CoV-2 in wastewater. Importantly, we discuss factors affecting the quality of wastewater surveillance ranging from temperature, pH, starting concentration, as well as the presence of chemical pollutants. These factors greatly affect the reliability and comparability of studies reported by various communities across the world. Overall, this review provides a broadly encompassing guidance for epidemiological study using wastewater surveillance.

1. Introduction

Coronaviruses (CoVs) are a group of enveloped single-stranded positive-sense RNA viruses, distinct for its club-like spikes proteins that project from their surface [1,2]. These surface proteins are proteins involved in the virus' life cycle of assembly, budding, envelope formation, and pathogenesis [2]. CoVs can be classified into two main groups according to their host targets – animal and human coronaviruses. Human coronavirus (HCoV) was first identified in the mid-1960s. To date, there are seven HCoV types that are infectious to humans. Four of these types (HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1) cause only mild diseases and the common seasonal cold [3–5]. Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are two HCoVs that caused severe outbreaks in 2002 and 2012, respectively.

More recently, the emergence of the Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) in late 2019 has caused a global health pandemic. On January 30, 2020, the 'Coronavirus Disease 2019' (COVID-19) pandemic was declared as a Public Health Emergency of International Concern by the World Health Organization (WHO) [6]. As its name suggests, SARS-CoV-2 is closely related to SARS-CoV in terms of its genomic and structural composition, but it is far more highly transmissible [5]. According to USA health protection agency, Centers for Disease Control and Prevention (CDC), transmission routes of SARS-CoV-2 are categorized as inhalation of virus, deposition of virus on exposed mucous membranes, and touching mucous membranes with soiled hands contaminated with virus [7]. Among these, airborne transmission is now thought to be the primary transmission route of SARS-CoV-2 [8]. It spreads primarily through droplets generated when an infected person coughs or sneezes, or through droplets of saliva or discharge from the nose [9]. The rapid spread of SARS-CoV-2 is further worsened due to globalization and increased human interactions [10]. The exponential rise in coronavirus transmission have caused massive lockdowns, from city to national levels, to alleviate the spread of SARS-CoV-2. Donning of personal protective equipment such as surgical masks and N95 were also encouraged in increased risks areas such as hospitals and patient care facilities to contain the virus [11,12]. The pandemic has thrown up a lot of fresh research in different areas from the understanding of how masks serve to protect us and our surrounding people from viruses, to how sanitizing agents can help in preventing transmission through the fomite route, to the study of how aerosols facilitate the spread of the virus, to the design of a smart mask that can observe a patient's health conditions during infection [13–22].

A COVID-19 patient may display clinical symptoms that were similar to that of SARS-CoV infections, including fever, fatigue, dry

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cough, myalgia, and gastrointestinal infection symptoms [23,24]. Up to 33% of COVID-19 patients, with a staggering 76%–86% of critically ill patients, has reported gastrointestinal symptoms [25]. These symptoms include diarrhea and vomiting. Due to diarrhea and vomiting caused by gastrointestinal infections, viral RNA is shed and disposed in wastewaters [26]. The presence of viral RNA in wastewater highlights a potential indirect infection pathway, which if left unexamined, can cause serious public health risks. While wastewaters may not be a primary source of transmission of CoVs, there is still an urgency to understand and evaluate this possible mode of transmission and its possible benefits as a community health surveillance tool. This research area was first highlighted in the SARS-CoV-1 outbreak. During the SARS-CoV-1 epidemic in 2003, diarrhea was a common presenting symptom [27,28]. The significant number of gastrointestinal infections would mean a substantial load of viral RNA in the sewage. It was reported that there was an outbreak within an apartment building where sewage could possibly be the virus' transmission source [29]. It has been suggested that toilet flushing, and faulty plumbing systems led to the spread of the virus in the community via the building's wastewater systems [29,30].

Given the potential severity of this infection's pathway whilst taking lessons learnt from the SARS-CoV-1 epidemic, a systematic quantification and detection mechanism for SARS-CoV-2 in wastewaters would be beneficial in mitigating transmissions through wastewaters. Several papers have discussed the occurrence, persistence, and removal of SARS-CoV-2 in wastewater. Kitajima and colleagues [26] discussed the importance that wastewater surveillance provides to understand the epidemiology of COVID-19 and the potential role that quantitative microbial risk assessment (QMRA) plays in reducing the impact of the current COVID- 19 outbreak. Foladori and colleagues [31] discussed the methods for identification and concentration of SARS-CoV-2 from wastewater. They also highlighted the areas where further research is needed in terms of sampling and identification of SARS-CoV-2 in feces and wastewater and studies on the possibility of faecal-oral transmission. Silverman and Boehm [32] provided a systematic review on the effect of disinfectants on the decay rates of human coronaviruses in water and wastewater.

Given the ongoing pandemic, the progress in wastewater-based epidemiology (WBE) as a useful surveillance tool for viral pathogens, in particular, SARS-CoV-2, will be discussed in this review paper. It is also apt to take stock of the different methods and policies of how countries are using wastewater to understand the spread and presence of COVID-19 cases will be discussed. In addition, this review seeks to provide an assessment of wastewaters as a transmission pathway of SARS-CoV-2. The viability of wastewater surveillance methodologies for SARS-CoV-2 detection will be discussed, along with the factors that may affect its accuracy and effectiveness. Disinfection methods, which are of particular importance in wastewater treatment plants, will also be discussed for SARS-CoV-2 containment. With these, a holistic understanding and evaluation of WBE as an approach for infectious disease surveillance and the public health risks associated with SARS-CoV-2 in wastewater can be achieved.

2. How does SARS-CoV-2 ends up in wastewater: gastrointestinal shedding and time lag

SARS-CoV-2 positivity can be observed in feces of persons who are symptomatic and asymptomatic [31,33]. In some cases, viral shedding of SARS-CoV-2 in the feces was still present up to 10 weeks after respiratory clearance and throat swabs and urine samples gave negative detection [34–38]. Human fecal matter inevitably ends in the sewage systems which can provide an ideal condition for enteric virus to replicate and spread through viral loaded aerosols. It is also due to this unrestricted entry of wastewater into the environment and the transportation of microbial contaminants to humans and organisms that makes it a major source of pathogen transmission. SARS-CoV-2, shed via human excreta, can slowly find its way to the community wastewaters and environment. For poor basic sanitation settings such as polluted waters and inadequate sewage, catastrophic outcomes may occur, if environmental surveillance is not in place [39].

WBE is an environmental surveillance approach that was first implemented to systematically analyze chemical residues in wastewater influent to measure a population's consumption of or exposure to chemicals such as legal and illegal drugs of abuse [40–42]. Beyond the testing of drugs and chemicals, WBE has the potential to act as a complementary approach for current infectious disease surveillance systems and as an early warning system for disease outbreaks [43,44]. WBE analysis of population pooled wastewater is an attractive candidate for monitoring public health as it provides efficient, comprehensive and real-time monitoring data [43]. In past years, WBE is commonly used for detection of non-enveloped viruses (which lack an outer lipid bilayer covering) such as polioviruses, adenoviruses, and coliphage [45]. WBE is now seen as a viable method for early monitoring and detection of SARS-CoV-2, an enveloped virus.

Countries such as Czech Republic [46], Italy [47], Spain [48,49], Japan [50], Qatar [51], Hungary [52], Canada [53,54] and Iran [55] have studied the viability of WBE analysis for SARS-CoV-2. Samples of untreated and treated wastewater were collected from the countries' wastewater treatment plants (WWTPs). These tests have demonstrated that SARS-CoV-2 can be detected and quantified in wastewaters, showing the feasibility of WBE. In Spain, SARS-CoV-2 was detected in a sewage sample 41 days before their first reported COVID-19 case [48]. Wastewater surveillance also proved to anticipate the onset of the second wave in Spain, leading to the implementation of efficient lockdown measures, alleviating the pandemic situation. Wastewater surveillance conducted in Hungary was also effective in predicting the second wave of virus outbreak, proving to be a useful and cost-effective tool in outbreak detection [52]. Samples were taken from WWTPs servicing the entire population in the capital, Budapest. Similarly, in Canada, WBE was used to monitor the public health in the city of Halifax and Ottawa. allowing health authorities to detect the early rise of cases in Halifax and the resurgence of cases in Ottawa [53,54].

Apart from WWTPs, Albastaki et al. [56] and Ahmed et al. [57] has studied the use of WBE in airline and cruise ship sanitation systems for SARS-CoV-2 detection in the United Arab Emirates (UAE). Both group of researchers successfully detected SARS-CoV-2 RNA in the wastewater samples. However, virus concentration methods have to be improved as concentration were found to be near assay limit of detection [57]. In contrast to large community testing, WBE can also be used to test a small community for the presence of the virus. In Singapore, WBE was used as a form of non-intrusive surveillance method to monitor SARS-CoV-2 in residential blocks [58]. Despite the absence of confirmed COVID-19 cases, increased frequency and concentration of SARS-CoV-2 was detected in the wastewaters. This allowed effective measures of subjecting residents to Polymerase Chain Reaction (PCR) tests, which yielded a positive COVID-19 case, affirming the wastewater testing results.

In the USA, wastewater surveillance was conducted in the universities as students return to campuses in the Fall Semester of 2020 [59,60]. The University of Arizona was able to use WBE to detect, identify and isolate three infected individuals which helped to swiftly avert potential disease transmission in the campus [59]. At the University of North Carolina at Charlotte, wastewater

surveillance enabled the identification of asymptomatic COVID-19 cases, which could not be detected through the other campus monitoring programs [60]. This is a successful cost-effective strategy to mitigate COVID-19 outbreaks, considering the substantial population of students living in on-campus dormitories. Through these studies, it is well-agreed that wastewater surveillance has the potential to act as a complementary strategy with clinical testing to maximize the probability of detecting COVID-19 cases in the community [48,57,58,61–63].

The wastewater samples collected from the various as-mentioned sources has to first be concentrated for the virus before diagnostic tests can be run. Using the samples collected from WWTPs as an example, the recovery, detection and quantification of SARS-CoV-2 from wastewaters is illustrated in Fig. 1. This wastewater, containing the viral load of SARS-CoV-2, flows via a series of sewage networks from domestic, industrial, and commercial sites into WWTPs, where the untreated wastewater will be commonly collected [64]. Meandering through a complex network of drains and sewage increases the challenges of SARS-CoV-2 detection process and may result in a time lag in which tests can be done to effectively trace enveloped SARS-CoV-2 from the community. As such, it has been suggested that the monitoring and recovery of SARS-CoV-2 in sewage, before reaching a WWTP, can be exploited [31].

For conventional recovery of untreated wastewater, there is a challenge in the viral load due to dilution of feces upon entering WWTPs. Despite the lower concentration of viral SARS-CoV-2, typical WWTPs generally do not remove virions completely [29]. Coupled with high influent viral loads during pandemics, this issue will lead to inadequate removal of viruses before water discharge from WWTPs [29] into the community via sources of drinking water supply and recreational venues such as swimming pools. From wastewaters to WWTPs, SARS-CoV-2 can survive in stool specimens for an average of 22 days [65]. When viruses have a survivability rate (i.e. T_{90} - time required to reach 90% inactivation) of hours or days, there is a high possibility of the virus reaching the WWTP [31]. Compared to recovery of non-enveloped viruses, there is scarce information on the recovery of enveloped viruses (in particular, SARS-CoV-2) from wastewaters. There are many challenges on its recovery as enveloped viruses are less stable in wastewaters compared to non-enveloped viruses [66–68] and tend to be sensitive to some organic solvents [69]. The factors which affect SARS-CoV-2 survival time and its recovery from wastewaters will be discussed in **Section 4**.

3. Wastewater viral detection Methods

3.1. Pre-treatment and culture

After the recovery of wastewater, it must be processed and analyzed for the presence of SARS-CoV-2 viral RNA. Current SARS-CoV-2 WBE studies used distinct sampling methods, viral concentration methods, real-time RT-PCR (real-time Reverse Transcriptase Polymerase Chain Reaction) targets, and process controls and criteria [39]. First, the collected wastewaters need to be stored at low temperatures (usually at 4 °C) in order to preserve the viral load and viability. As investigated separately by Medema et al. [70] and Cutrupi et al. [71], viral RNA copies did not decrease significantly when stored at 4 °C for 14 days.

Chemical and thermal pre-treatments are usually carried out to inactivate the virus thus providing safer conditions during sample handling. Much research on optimal thermal pre-treatment procedures for SARS-CoV-2 has been conducted however, a drawback of utilizing thermal pre-treatments is that the viral RNA load will resultantly be lower [71,72]. Discussion on thermal inactivation will be elaborated in **Section 4.** To chemically inactivate SARS-CoV-2 for safer sample handling, Monteiro and colleagues evaluated enzymatic (nuclease) and viability dye (Reagent D) pretreatments to porcine epidemic diarrhea virus (PEDV) as a CoV surrogate [73]. Molecular approaches such as quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) were utilized to detect SARS-CoV-2 infectivity in treated wastewaters. SARS-CoV-2 infectivity tests were performed on infectious and heat-inactivated PEDV, and between infectious and heat-inactivated PEDV following the two pre-treatments. No differences between nuclease-treated infectious and heat-inactivated PEDV were found

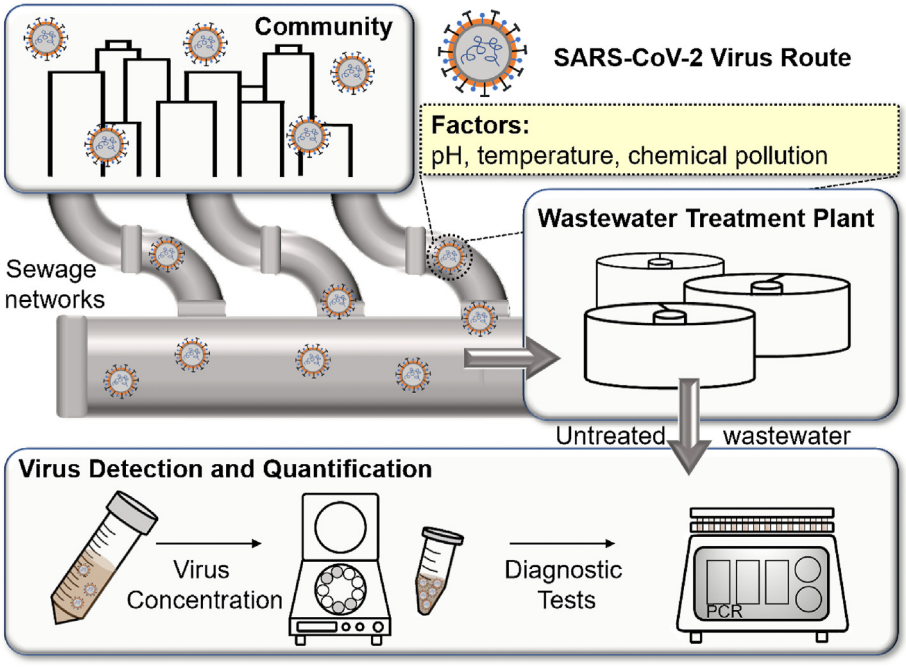

Fig. 1. SARS-CoV-2 detection and quantification from wastewater sources as an infectious disease surveillance system for communities.

while Reagent D was able to significantly decrease the RT-qPCR signal of heat-inactivated PEDV. Auerswald and colleagues performed chemical inactivation of SARS-CoV-2 by using AVL buffer including carrier RNA; AVL buffer, GeneReach sample (contains guanidinium thiocyanate (GITC) and t-Octylphenoxypolyethoxyethanol (Triton X-100)) and Formaldehyde in PBS [74]. It was found that all inactivation methods can successfully reduce viable SARS-CoV-2 to undetected levels.

After the pre-treatments, cell culture studies can be conducted in order to determine how long the virus can survive in different storage environments. Mammalian cell line such as Vero E6 cell culture are commonly used to determine the viability of SARS-CoV-2 in the wastewater samples has been investigated by a few groups of researchers [73,75–77]. However, as there are challenges to virus isolation from cell cultured systems of SARS-CoV-2 from wastewater [73,78], the need to examine the stability of SARS-CoV-2 wastewater samples in different environmental conditions needs to be discussed.

3.2. Current methods of detection

Diagnostic tests have been developed for SARS-CoV-2 and they are based on three broad techniques - serological, molecular, and point-of-care detection techniques [79]. First, SARS-CoV-2 serology (also known as antibody) testing look for antibodies in a sample to determine if an individual has had a past infection with the virus that causes COVID-19 [80]. A serological test identifies an individual's antibody immune response against a specific past or current infection. The test detects the immune response through antibodies (such as Immunoglobulins IgG, IgM and IgA) in a COVID-19 patient's serum and plasma [81]. As such, the test is also unable to detect the early stage of infection. CDC advised that serologic tests are designed for surveillance and research purposes, and detects previous infections in people who had few or no symptoms [82]. A retrospective clinical evaluation on the performance of 10 commercially available rapid diagnostic tests was carried out by Dortet and co-workers. This evaluation was designed using the 2015 Standards for Reporting of Diagnostic Accuracy Studies [83]. Their findings highlight the need for carefully verified assays and appropriately designed serological studies to characterize transmission dynamics, refine disease burden estimates, diagnose suspected cases, and confirm clinically diagnosed patients without access to RT-PCR [84].

Second, molecular approaches of COVID-19 detection refer to diagnostic tools that can detect the single stranded, positive-sense RNA virus. An important step in nucleic acid detection tools is in the amplification of the target sequence, especially when there is often a limited amount of DNA available [85]. RT-PCR and isothermal nucleic acid amplification are two well-developed amplification methods diagnostic tools. RT-PCR tests, as one of the most used SARS-CoV-2 detection methods [86,87], will be further described in Section 2.3. Despite the wide adaptation of PCR, PCR machines are costly, and its thermal cycling process is time consuming. The drawbacks of PCR led to the development of alternative amplification methods such as isothermal amplification as promising candidates for SARS-CoV-2 detection. Isothermal techniques such as Loop-mediated Isothermal Amplification (LAMP), Nucleic Acid Sequence Based Amplification (NASBA), Sequence Mediated Amplification of RNA Technology (SMART), Strand Displacement Amplification (SDA), and more recently Multiple Cross Displacement Amplification (MCDA) have been developed [79]. Compared to PCR, these isothermal nucleic acid amplification detection methods take place over a constant temperature. The absence of temperature cycling or rapid heating and cooling mechanisms makes it an efficient, simpler, and rapid detection tool which can be used in resource-constrained areas [85,88].

In addition, amplicon-based metagenomic sequencing is an effective way to target and identify the organisms of interest. For instance, “Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) -associated protein” systems or CRISPR-Cas systems can be used to detect amplicons are generated from the viral RNA of SARS-CoV-2. The advantage of CRISPR-based nucleic acid detection tools is that they can be programmed for repurposed applications and function in just minutes under mild conditions. With this pathogen detection method, researchers can sample the genes in SARS-CoV-2 present in each complex sample. However, there are still challenges such as the need for improved specificity due to inherent off-target effect [89–92]. Recent work studied the coupling of CRISPR/Cas system with nanotechnology-based approach for colorimetric detection of SARS-CoV-2. Combining the advantages of exponential amplification or loop-mediated isothermal amplification (LAMP) from colorimetric detection method and the target-specific *trans*-cleavage from CRISPR-based nucleic acid detection method, Cao and colleagues have developed an assay that provides sensitive and specific detection of SARS-CoV-2 RNA within 1 min [93].

Third, point-of-care testing refers to diagnostic of patients with the lack of access to centralized lab facilities. Chip-based biosensors of lateral flow assays are one example of point-of-care test developed for SARS-CoV-2 detection. Using a patient's urine or blood sample, lateral flow assays comprising of gold nanoparticles conjugates and capture antibodies on two constituent lines. Red lines represent the presence of only gold nanoparticles, and blue lines represent a clustered gold solution on account of plasmon band coupling [79]. Gold nanoparticles aggregate rapidly and irreversibly change in color from red to purple due to antibody-antigen interactions [94,95]. This rapid diagnostic tool is easy and convenient to use. However, these tests are for single usage and have poorer analytical sensitivity compared to RT-PCR [79]. Apart from lateral flow assays, there are recent developments of material-based biosensors such as Field-Effect Transistor-Based biosensors [96,97] and electrochemical-based biosensors [98–100] as potential detection tools for SARS-CoV-2.

Despite the many advantages, the aforementioned diagnostic tools are not commonly found in local clinics. As such, clinics would need to send samples to clinics with such diagnostic capabilities, resulting in delayed test results. To this end, several rapid antigen tests (RATs) have been developed and commercialized for quick screenings of individuals to detect possible infection. RATs detect SARS-CoV-2 viral proteins (antigens) in respiratory tract specimens. These self-tests kits are easy to be administered and results could be obtained in a mere 15–30 min [101]. These tests are especially beneficial to screen asymptomatic individuals and should be encouraged to as a requirement before mass gatherings in offices, dining and concerts. Stohr and colleagues evaluated the performance of two RATs - BD Veritor System and Roche SARS-CoV-2 [102]. The sensitivity and specificity of these self-testing kits were compared to qRT-PCR. Through their study, it was found that specificity was extremely high (>99%) whereas sensitivities were 76.1% (BD Veritor System) and 80.1%. (Roche SARS-CoV-2). It was also found that the sensitivity varies across the age groups, with the sensitivity higher in younger individuals. Sakai-Tagawa and colleagues evaluated 27 RATs commercially sold in Japan and found that only 9 RATs (ESPLINE SARS-CoV-2, ALSONIC COVID-19 Ag, COVID-19 and Influenza A + B Antigen Combo Rapid Test, ImmunoArrow SARS-CoV-2, Fuji Dri-chem immune AG cartridge COVID-19 Ag, 2019-nCoV Ag rapid detection kit, Saliva SARS-CoV-2 (2019-nCoV) Antigen Test Kit, and Rabliss SARS-CoV-2 antigen detection kit COVID19 AG) showed high sensitivity to the B.1.617.2 (Delta) variant [101]. However, it was noted that RATs may potentially give

negative results for test samples when the concentration of virus in the sample is low. This could result in untimely treatment to patients and ineffective mitigation on the spread of the virus. Therefore, it is much needed that the ideal detection method should be sensitive, specific, rapid, portable, repeatable, cost-effective, and easy to use. With the recent emergence of B.1.1.529 (Omicron) variant, there is an urgent need to conduct clinical studies to ensure the suitability and sensitivity of the various RATs in the market.

3.3. PCR test for RNA correlation

After the collection and concentration of SARS-CoV-2 from wastewater, quantification measurements are the final stages for the detection of viral RNA. As the most adapted diagnostic test for pathogens, PCR tests are used to detect COVID-19 virus as well. The concentrated virus samples contain a human's genetic material and viral RNA, if present. For a PCR technique, RNA is reverse transcribed to DNA using a specific enzyme. RT-PCR allows the use of RNA as a template. Hospitals have adapted the RT-PCR tests for SARS-CoV-2 diagnosis [103,104]. The RNA template is amplified though multiple thermocycling processes. Each thermocycling process consists of three main steps of denaturation, annealing and elongation. The RT-PCR process creates billions of copies of the viral RNA from each viral RNA strand, to accurately detect the presence of COVID-19 in a person.

4. Concentration, viability, and time decay of viruses in wastewater

4.1. Quantitative methods for studying and correlating wastewater quality analysis to outbreak

One of the challenges of utilizing WBE for SARS-CoV-2 detection from wastewaters is in its relatively low viral particle concentration in wastewaters [105]. Detection of SARS-CoV-2 RNA in wastewater and wastewater aerosols does not necessarily indicate viability and infectivity of the viral particles [31]. The viral load in wastewater is significantly lower as compared to viral load in feces. This is due to the high turbidity of raw wastewater which may interfere with molecular methods of assaying for viruses [106–109]. Also, viruses tend to be adsorbed on the surface of flocs in sludge. These characteristics affect the accuracy of the detection of virus in these samples. Therefore, viral concentration methods are necessary for viable quantification, as illustrated in Fig. 2. However, the results only determine the cytopathogenic effect of the infected sample and does not provide complete information on the infectivity of viruses present in water media. Hence, the challenge remains to preserve the viability of the virus during the sampling, the handling and the treatment of the wastewater sample.

Concentration methods fall under four main categories: two-phase separation/partition precipitation (such as PEG-based separation), particle exclusion, VIRuses ADsorption–ELution (VIRADEL), and ultrafiltration [105]. However, these concentration methods were developed and optimized for non-enveloped enteric viruses. The utilization of these methods for enveloped viruses (including CoVs) results in lower recovered concentrations [69,105,111]. As such, there is a research gap on the recovery efficiencies for enveloped viruses, which has differing structural and physical characteristics of the viruses compared to non-enveloped viruses [26,112]. Several research groups have proposed concentration of SARS-CoV-2 from wastewater samples from methods such as PEG-based separation methods, ultracentrifugation methods and electronegative filtration methods. The various concentration methods and process steps employed to concentrate SARS-CoV-2 from wastewaters is tabulated as Table 1.

Ultrafiltration, polyethylene glycol (PEG) precipitation, ultracentrifugation, and filtration with an electronegative membrane have been used for concentrating viruses in wastewater to enhance the usefulness of detection assays. Although reports suggest that absorption-extraction methods are more efficient, the efficacy in virus concentration can be hindered due to turbidity of the

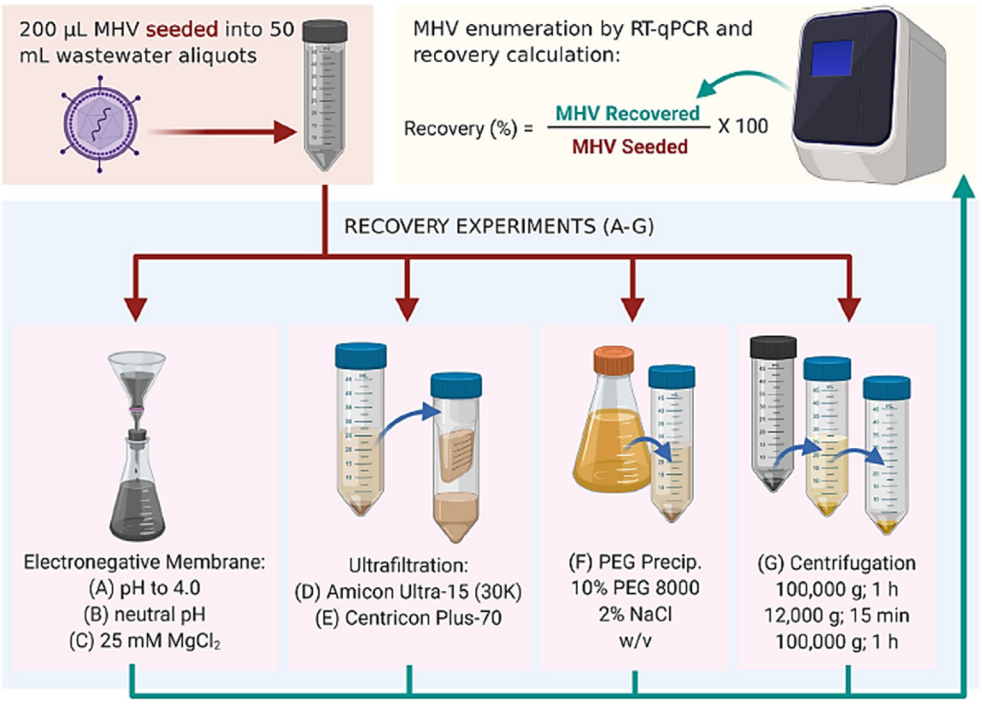

Fig. 2. Typical virus concentration processes to concentrate Murine Hepatitis Virus (MHV), a surrogate for SARS-CoV-2, from wastewater. Figure reproduced with permission from Ref. [110]. Copyright Elsevier 2020.

Table 1
SARS-CoV-2 concentration methods from untreated wastewater samples.

Concentration method	Process steps	Ref.
Two-phase separation method	- Flocculation using beef extract solution in glycine buffer - Acidified and beef extract flocculated by addition of HCl - Suspension stirred for 10 h - Centrifugation at 1000*g* for 30 min at 4 °C - Pellet dissolved in phosphate-buffered saline	[46]
Two-phase separation method (PEG-based separation)	- Centrifugation at 4500*g* for 30 min - Filtration for supernatant using 0.22 μm filters - Addition of PEG and NaCl - Incubated overnight at 17 °C and 100 rpm - Centrifugation at 1300*g* for 90 min - Pellet resuspended in RNase-free water	[113]
Two-phase separation method (PEG-based separation)	- Filtration via 0.20 μm membrane - Addition of PEG-8000 and NaCl Centrifugation at 12000*g* for 2 h or until pellet is visible	[114]
Two-phase separation method (PEG-based separation)	- Centrifugation to remove large particles - Addition of PEG or alum and centrifuged - Incubated at 4 °C at 100 rpm for 12 h - Centrifugation at 14000*g* for 45 min at 4 °C - Virus suspended in phosphate-buffered saline - Filtered through 0.22 μm filter - Centrifuged using 30 kDa ultrafiltration membrane	[115]
Ultrafiltration	- Centrifugation at 4654*g* for 30 min to remove large particles - Supernatant filtered through 100 kDa Centricon® Plus-70 by centrifugation at 1500*g* for 15 min	[70]
Ultrafiltration	- Centrifugation at 3000*g* for 30 min to remove large particles - Supernatant filtered through 100 kDa Centricon® Plus-70 by centrifugation at 1500*g* for 15 min - Filter unit inverted and centrifuged at 1000*g* for 2 min	[116]
Ultrafiltration	- Filtered through 20 μm, 5 μm and 0.45 μm membrane filters - Concentrated using 100 kDa Corning Spin-X concentrators - Extracted with RNeasy Mini Kit and RNase free buffer	[117]
Ultrafiltration	- Centrifugation at 200000*g* for 1 h at 4 °C - Viral pellets resuspended in phosphate-buffered saline - Viral concentrate was lysed and extracted - Extracted nucleic acids filtered through PCR inhibitor removal kit	[118]
VIRADEL	- Addition of $MgCl_2$ - Sample passed through 0.45 μm pore size electronegative filter - Removal of Mg ions through H_2SO_4 - Sample eluted with NaOH and recovered with tube containing H_2SO_4 and Tris-EDTA - Centrifuged using 30 kDa ultrafiltration membrane	[116]
VIRADEL	- pH adjusted to 6.0 and $Al(OH)_3$ precipitate formed by adding $AlCl_3$ solution - pH readjusted to 6.0 and mixed - Centrifugation at 1700*g* for 20 min - Resuspension of pellet in beef extract at pH 7.4 - Centrifugation at 1900*g* for 30 min	[119]

wastewater. An effective method to solve the turbidity of wastewater would be centrifugation to remove larger particles and debris coupled with Polyethylene glycol (PEG) precipitation [120–122]. It is a simple and reliable methodology which can handle large volume of wastewater and is inexpensive for the concentration of the SARS-COV-2 [123]. Different versions of PEG precipitation have been used for the assessment of SARS-CoV-2 in untreated wastewater, but the efficiencies were not reported [124]. That said, there are reports on results from Pre-PEG and Post-PEG precipitation which were validated using a qRT-PCR assay which showed that PEG precipitation resulted in an increase of genome equivalent copies from 1.4×10^8 to 4×10^9 copies/mL derived from tissue culture cell supernatant [125]. This concentration yield is viable for virological assays involving high Multiplicity of Infection (MOI) in several human cell lines.

4.2. First order decay rate constants

Stability of the genome in wastewater is reported by Aaron Bivins and co-workers. They applied the PEG precipitation methodology to investigate the first order decay of infectious Covid-19 strain with initial titer of 10^5 and 10^3 median tissue culture infectious dose per milliliter ($TCID_{50}mL^{-1}$) and assess its fate and stability under room temperature and alleviated temperature conditions with wastewater as the medium. The infectious SARS-CoV-2 is observed to persist in wastewater for maximum of up to 7 days in room temperature and less than 10 min in temperatures above 50 °C. The RNA component of the virus strain persists much longer periods under ambient and elevated temperature conditions [78]. Despite these observations, it still does not address the effective relationship between viral titers and COVID-19 incidence due to the loss of real-time data and viability of virus infection from concentration and processing methods that are necessary for tests and analysis. La Rosa et al. summarized that the virus has low scale water borne infectivity and at present there is no current evidence of the virus transmitting through contaminated water [126]. However, it is good to note that aerosols from wastewater treatment processes could carry infectious agents, including respiratory viruses, which may risk viral infections to the wastewater workers [127]. A recent study reported that SARS-CoV-2 aerosols could maintain their infectivity for up to 16 h [128]. Another study to mitigate aerosol transmission of Covid-19 was carried out by Suwardi et al. As it is less likely to detect the presence of airborne virus in air, they proposed a two-throng method that utilizes negative air ions (NAIs) that are generated by Plant-Based Ionizers to mitigate Covid-19 transmission by aerosol. The aerosols attached with NAIs becomes heavier and tend to be attracted to positive charged surfaces to form fomites [129].

Quality data is limited by the wastewater journey and accuracy can be fine-tuned by identifying potential sites based on blueprint of pipelines and sewage to determine and quantify the source of infectious SARS-CoV-2. Additionally, wastewater sample storage and handling conditions prior to analysis could also create bias in viral quantification. Early detection of variants within a localized vicinity through wastewater surveillance can serve as an indicator to the increasing or decreasing trends in infection cases, providing guidance for policymakers as they trace infection clusters and consider public health measures. Therefore, quantifying the decay rate of SARS-CoV-2 RNA is important for WBE because decay in the RNA signal between the time of excretion in feces and the time of sampling from the wastewater collection system may lead to systematic bias in subsequent estimates relevant to public health. The source of wastewater is also a crucial factor for the fate of the virus. It has been postulated that SARS-CoV-2 may be sensitive to low pH, with more research needing to be carried out to determine the effect of acidification on SARS-CoV-2 recovery from wastewater. In addition, chemical oxygen demand, flow rate, ammonia, pH, permanganate value, and total solids are wastewater characteristics to be considered. Among the parameters assessed using the Adaptive Neuro-Fuzzy Interference System (ANFIS) model, ammonia and pH showed significant association with the concentration of SARS-CoV-2 RNA measured. Increasing ammonia concentration was associated with increasing viral RNA concentration and pH between 7.1 and 7.4 were associated with the highest SARS-CoV-2 concentration.

5. Factors affecting wastewater study as proxy for COVID-19 epidemiology

5.1. Starting concentration and presence of chemical pollution

To begin exploring the study of wastewater as a proxy for COVID-19 epidemiology, a universal standard needs to be achieved in sample collection, treatment, and testing to account for the disparity in wastewater conditions at varying locations. Following studies which prove that the SARS-COV-2 mRNA can be detected via rectal swabs and sampling of feces [130,131], it may be intuitive to take advantage of wastewater and knowledge of the sewage network as a surveillance method of the virus in the region. An additional advantage being that, up till date, active replication of the virus in stool was reported to be absent or minimal [132]. Hence, by quantitatively studying the virus in wastewater, an estimation of the number of infected individuals can be calculated and mitigation efforts can be appropriately formulated in response.

However, the initial challenge faced is that feces will be combined with other waste products and heavily diluted upon entering the sewage system, greatly diminishing the detected viral load per volume. The concentration of the virus in wastewater is reported to decrease by at least 4 orders of magnitude in comparison to direct sampling [31]. According to a study conducted by Cheung et al. the median viral load detected in the stool samples of COVID-19 positive patients were $10^{5.1}$ copies/mL for individuals with diarrhea and $10^{3.9}$ copies/mL for those without the symptom [133]. Comparatively, a time-course study by Wurtzer et al. conducted at the time of rapid spread reported the viral load in Paris across 3 WWTPs to be in the range of $50-3 \times 10^3$ copies/mL (calculated in the review by Foladori et al.) [31,134]. In the same time frame, Randazzo et al. reported an average viral load of 2.5×10^2 copies/mL across 6 WWTPs in Spain [135]. This dilution is contributed by a few factors including the variations in daily water discharged by the household, rainwater and parasitic inflow into the sewer network. Furthermore, the pandemic has caused an increase in water consumption due to heightened hygienic concerns which adds on to the dilution of viral concentrations in wastewater [136].

It is important to note that the method of sample collection may also present discrepancies in the viral concentrations. Grab sampling of wastewater may lead to inconsistent results as temporal variations exist in viral concentrations, depending on the duration of infection. The highest concentration of viral mRNA was noted to occur during the first week from the onset of symptoms at 10^7 copies/mL. Subsequently, a decrease by 2–3 orders of magnitude is observed in the following weeks as reported in the study by Wolfel et al. [132]. In contrast, composite samples which are collected by averaging the results of multiple aliquots in a given time period, may provide a more representative data.

As mentioned, various works have developed multiple methods of molecular detection of SARS-CoV-2 from wastewater samples which were briefly summarized by Kitajima et al. [26]. Ultimately, the concentration of viral mRNA detected per day is tabulated for a period to study the spread of the virus. The daily viral load in wastewater is calculated by measuring the concentration of SARS-CoV-2 mRNA from the wastewater samples (in copies/m^3) and multiplying it with the daily flow rate of wastewater (in m^3/d). Taking into consideration the dilution of viral concentrations in wastewater, this result can be compared to the viral load detected in stool samples of clinically diagnosed COVID-19 individuals. Subsequently, an estimate can be made on the number of infected persons in the catchment area. For example, a study by Ahmed et al. utilizing Monte Carlo simulation yielded reasonable estimates on the number of infected individuals when compared against clinical observations [137].

Owing to the limited knowledge of the novel coronavirus while establishing the basis for wastewater studies, the behavior of SARS-COV-2 is estimated to mirror other CoVs. There is evidence that enveloped viruses are more susceptible to environmental factors than their non-enveloped counterparts and their infectivity in water is greatly diminished once the lipid envelope is damaged [138,139]. It is generally understood that chemical pollution can denature the virus and greatly decrease its viability [140]. The rate of reduction may be further expedited in the presence of chemicals typically used in disinfection such as chlorine-based chemicals, organic solvents, highly acidic or alkaline solutions [139]. This also translates to the virus being viable for a longer time in purer waters. For example, a study conducted by Casanova et al. found that the infectivity of coronaviruses is reduced twice as quickly in sewage in comparison to reagent-grade water [141]. Quantitatively, Gundy et al. established that a 99.9% decline in the viral load present in wastewater is observed after 2–4 days [142].

5.2. Temperature

The environmental temperature is also an affecting parameter in the viability of COVID-19 RNA in wastewater. In general, viruses persist for a longer time in lower temperature while elevated temperatures can cause increased sensitivity to other environmental factors and eventually lead to inactivation. Bibby et al. studied the persistence of SARS-CoV in wastewater using surrogates such as Phi6 and HCoV 229E and OC43. The study reported that surrogate coronavirus was detected in wastewater near room temperature (22–25 °C) for 2 days while it persists for 50 days at 4 °C [78]. Other studies similarly cited that SARS-CoV-2 is viable for 2 days at 20 °C and 14 days at 4 °C [26,136].

Following the extraction of wastewater samples, it is of best interest to transport the RNA-containing solution to laboratories without much deterioration in their state. The golden standard for storage and transportation of RNA samples is flash freezing, followed by further cryopreservation with nitrogen at −80 °C which has shown to maintain the stability of RNA for years [143]. For

shorter transportation durations, maintaining the solution at a low temperature using ice is recommended to reduce freeze-thaw cycles [144]. Many protocols developed in wastewater studies have retained the wastewater samples at 4 °C, presumably to maintain the viability of the sample [134,145]. Conversely, high temperatures promote the deactivation of the virus. In a model study conducted by Islam et al. the rate of inactivation of avian viruses AIBV and H9N2 was expedited with increasing temperatures. Both viruses were reportedly inactivated in 90 min at 55 °C or 15 min at 65 °C [146].

5.3. pH

Generally, ingested viruses are be destroyed by the low pH in the stomach. Some of the virus may be cloaked by surrounding food particles which protect them from the acidic environment and transporting them to the intestine [31]. While variations may exist in the pH sensitivities across the multiple types of viruses, it has been understood that SARS-COV-2 will be inactivated in extremely acidic or alkaline pH [146,147]. The response of COVID-19 to pH variations is modelled using other well studied coronaviruses such as SARS-CoV-1, MERS-CoV and IBV.

According to previous studies, the pH sensitivity of the studied coronaviruses show some degree of temperature dependence. Fig. 3 illustrates the findings from multiple sources as summarized by Cimolai [148]. It can be observed that the stability and viability of the selected model viruses vary across the pH scale with inactivation mostly noted at extremely low or high values at both 4 °C and 37 °C. In his review, aside from the surrogates mentioned in Fig. 3, Cimolai details the pH dependent activity of other surrogate viruses such as FCoV and CCoV at other temperatures which are observed to follow a similar trend [148].

Considering the effect of multiple environmental conditions on the veracity of viral loads in wastewater, it may be more useful to study the trend of viral decay or spread in the individual catchment areas instead of predicting number of infected individuals accurately. Fig. 4 depicts the expected data to be obtained from the collection and study of SARS-CoV-2 in raw sewage water in the described hypothetical example below.

Hypothetically, if there exist 2 individuals infected with COVID-19 in the catchment area (person A and B) on Day 1, the total concentration of viral RNA detected in raw sewage water is expected to be due to the additive contributions of both persons. However, the amount of virus shed per person varies due to the factors mentioned in the previous section. Sample collection, concentration and measurement continue multiple times a day, to obtain a composite sample representative of the daily viral load. Given that there are no new infections within a week, the viral load in wastewater is expected to decrease according to the decay rate.

However, if on another day, 2 new infections occur, the total concentration of viral RNA in wastewater will once again increase because of the 4 total infections. By plotting the total concentration detected daily, it is possible to study the spread of the virus in a given area in terms of new infections. Furthermore, relevant taskforces can adjust strategies accordingly to prevent further spread of the virus. A threshold concentration represented by the horizontal dashed line can be used as a reference to signal a new cluster of infections. Safe management measures such as social distancing and short-term quarantines can be imposed, and their effectiveness can be monitored via changes in the total concentrations.

Alternatively, from the concentrations detected, the amount of viral RNA shed can be obtained by extrapolation by 4 orders of magnitude to account for the dilution in sewage water [31]. Comparing these concentrations to those measured in stool samples in clinical studies, a rough estimate of the number of infected individuals can be demonstrated from these studies, similar to the

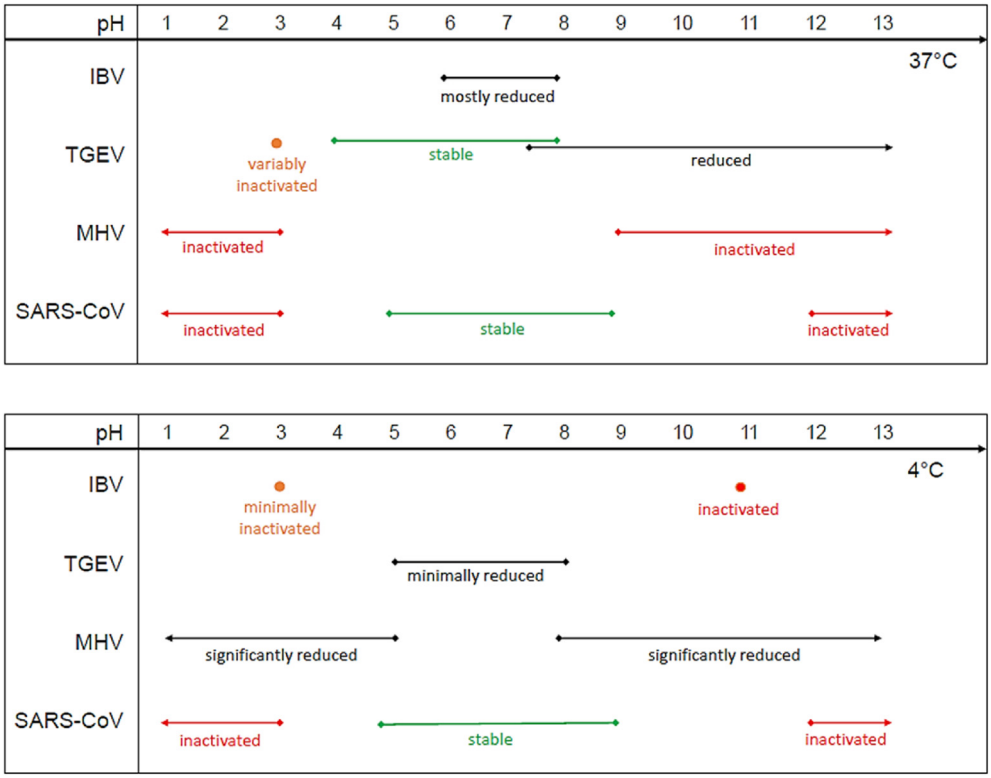

Fig. 3. pH sensitivity of viruses at 37 °C and 4 °C. Data obtained from Refs. [148–161].

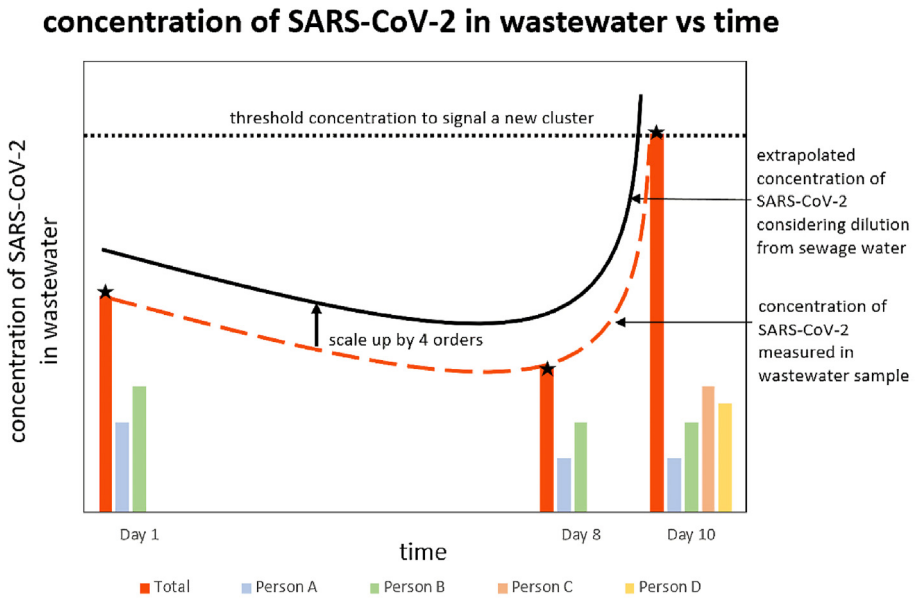

Fig. 4. An analogy of a possible data set collected by wastewater epidemiology of SARS-CoV-2. Viral load from wastewater concentration is calculated based on information and techniques used in Refs. [31,132,137]. The total concentration of viral RNA in wastewaters decreases with no new infections. An increase in total concentration of viral RNA in the wastewaters signify possible viral outbreak in community.

estimation by Ahmed et al. [137]. However, the focal point of this approach remains in analyzing the trend of viral concentrations to serve as a surveillance method and signal a potential outbreak in the near future. It is imperative to understand the type and accuracy of the data gathered in wastewater to assess the suitability of wastewater-based epidemiology. This is inclusive of the factors that affect the survivability of the virus in various environmental conditions.

6. Mitigation and disinfection Methods

6.1. Disinfection with ultraviolet

Sewage waters undergo multiple disinfection and purification steps at WWTPs to ensure that the resultant product is safe for use or consumption. Acknowledging the presence of SARS-CoV-2 RNA in raw wastewater, extra precaution is taken to ensure the removal of any virus remnants. According to the wastewater worker guidance release in February 2020 by the U.S. Occupational Safety and Health Administration (OSHA), effective disinfection methods utilized in WWTPs include oxidation with free chlorine and inactivation by ultraviolet irradiation (UV). These strategies are sufficient to safeguard the public and staff directly working with wastewater from the coronavirus [162].

The process of UV disinfection is described by Trojan Technologies [163]. Modern UV modules submerge UV lamps, encased in protective quartz sleeves into clarified wastewater for illumination. The dosage is monitored by UV intensity sensors and centralized control systems are used to vary the output of the lamps based on the volume and conditions of the wastewater for remote monitoring.

The large single-stranded RNA (ssRNA) genome (~29.8 kb) of SARS-CoV-2 likely renders it more susceptible to UVC compared to other enteric viruses [164]. Enveloped viruses such as COVID-19 do not seem to have a higher susceptibility to UVC (wavelength 200–280 nm) than non-enveloped viruses. This is because inactivation primarily targets the genome, while lipid membranes, the characteristic distinction identified for enveloped viruses, do not provide additional protection against such radiation.

Additionally, it is speculated that UVB (wavelength 280–315 nm) obtainable by exposure to natural sunlight, can increase the rate of inactivation of coronaviruses according to previous studies conducted on nonenveloped viruses [32]. It is generally believed that UV of wavelength 253.7 nm is optimal for ultraviolet disinfection, although few studies up till date have been cited to have used it specifically for treating COVID-19 infected waters [165]. The cost of investment and operation of UV disinfection systems are drastically cheaper in comparison to other methods and produces no toxic byproducts [166]. However, it has also been reported that disinfection with UVC can be limited by its shallow depth of penetration resulting in subpar results [167]. It was highlighted by Wigginton et al. that the attenuation of UV radiation through the solution should be well characterized to deduce the appropriate dose.

Previous work conducted for SARS-CoV reported a 400-fold decrease of infectious virus upon the exposure to 4016 μW cm^{-2}, 254 nm UVC for 1 min and subsequently full inactivation after 15 min [153]. With the development of UV based advanced oxidation technology, such as UV–H_2O_2, UV–Cl_2, UV–O_3 and UV–TiO_2, the possibilities of using reactive photolysis radicals to inactivate viruses is being increasingly explored [168].

6.2. Disinfection with chlorine

SARS-CoV-2 is known to be an enveloped virus, with a fragile outer membrane and is, therefore, less persistent in water. Its physical characteristics comprise of a lipid membrane that surrounds a protein capsule that consists of protein and glycoprotein. Chlorine can penetrate the lipid membrane and then reacts with the internal proteins which result in the deactivation of the virus [127,169,170].

A part of the work by García-Ávila et al. analyzed the resistance of SARS-CoV and phage f2 sown in domestic wastewater against different chlorine solutions [171]. Its analysis revealed that free

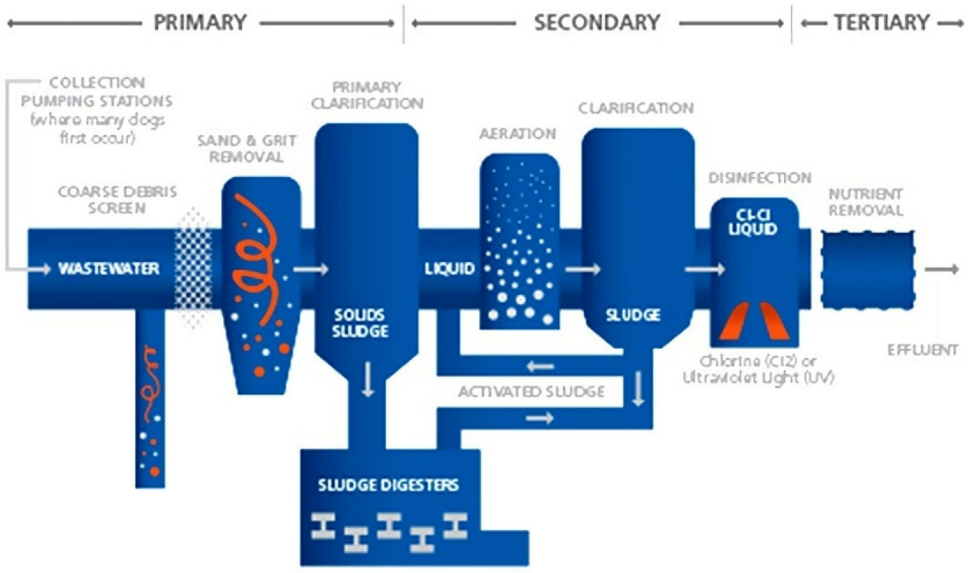

Fig. 5. Schematic diagram for wastewater treatment process. Figure reproduced with permission from Ref. [28]. Copyright Elsevier 2020.

chlorine is a more effective disinfectant compared to other chlorine-based disinfectants. By applying 10 mg/L of chlorine or 20 mg/L of chlorine dioxide, and after 30 min, the SARS-CoV was completely inactivated. Since the start of the pandemic, the drastic change in social behavior for personal and public hygiene has triggered an excessive use of chlorine-based disinfectant which can have a detrimental effect on the environment with high ecological risks. This data provides a useful guide for initiating protocols for prudent usage of chlorine-based disinfectants.

When it comes to wastewater treatment, the emphasis should be on the ideal doses of residual chlorine to effectively while efficiently disinfect water in the distribution system. Based on World Health Organization (WHO), the presence of residual chlorine of 0.5 mg/L, measured at the endpoints of the water distribution system, as shown in Fig. 5, must be guaranteed in all water systems for human consumption. To deter COVID-19, it is essential to ensure drinking water and wastewater services are fully operational. In addition to this is the responsibility of individuals to exercise proper personal hygiene and protective measures to mitigate the transmission of COVID-19 [172].

7. Conclusion and outlook

Wastewater surveillance is a critical part for the assessment and detection of pathogens and viruses, including the ongoing COVID-19 pandemic. The use of WBE as a community surveillance tool has been widely accepted and embraced by many countries. The non-intrusive nature and cost-effective epidemiological surveillance strategy allows WBE to be a convenient health monitoring and testing system of the community and helps to identify possible transmission channels. Wastewater surveillance is also a useful detection mechanism for assessing infected individuals who are asymptomatic, or throat swabs and urine samples gave negative detection. As more research studies emerge with the viability of wastewater surveillance for SARS-CoV-2 detection, it is of importance to consider the various factors that will affect the accuracy of wastewater testing methods. This is especially so, given the difference for each communities' wastewater environment and external conditions. With wastewater surveillance as a complementary strategy that prioritizes clinical testing and aids contact tracing efforts, better informed public health interventions will be implemented to alleviate the effects of the pandemic.

Concentration methods have also been established by various research groups showing the effectiveness of concentrating the virus for high accuracy of PCR tests from wastewater samples. However, concentration of SARS-CoV-2 is highly variable and decreases exponentially when diluted in municipal wastewaters. This causes a potential challenge for WBE analysis during early virus outbreaks. Apart from the initial virus concentration, SARS-CoV-2 is significantly affected by environmental factors such as the presence of chemical pollution, temperature and pH. Lastly, UV and chlorine has both shown capabilities as rapid mitigation and disinfection mechanisms for SARS-CoV-2 inactivation. However, precaution has to be taken to avoid exposure to excessive UV radiation on skins and eyes. Chemical disinfectants such as bleach and benzalkonium chloride may cause skin and eyes irritation as well. Although active chlorine is effective against coronaviruses, its effectiveness is limited by its poor stability. In this regard, the right concentration of the active ingredient and disinfectant contact time are essential for the efficacy of the disinfection process.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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